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Research paper

# Protective effect and mechanism of cannabidiol on myocardial injury in exhaustive exercise training mice

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ABSTRACT

Cannabinoid diphenol (CBD) is a non-toxic main component extracted from cannabis, which has the effects of anti-inflammatory, anti-apoptosis and anti-oxidative stress. In recent years, exercise-induced myocardial injury has become a research hotspot in the field of sports medicine and sports physiology. Exercise-induced myocardial injury is closely related to oxidative stress, inflammatory response and apoptosis. However, there is no clear evidence of the relationship between CBD and exercise-induced myocardial injury. In this study, by establishing an animal model of exhaustive exercise training in mice, the protective effect of CBD on myocardial injury in mice was elaborated, and the possible molecular mechanism was discussed. After CBD intervention, the arrangement and rupture of myocardial fiber tissue and the degree of inflammatory cell infiltration were reduced, the deposition of collagen fibers in myocardial tissue decreased. CBD can also significantly inhibit cardiac hypertrophy. Meanwhile, the expression of IL-6, IL-10, TNF-α, Bax, Caspase-3, Bcl-2, MDA-5, IRE-1α, NOX-2, SOD-1, Keap1, Nrf2, HO-1, NF-κB and COX-2 was recovered to normal. In addition, after CBD intervention, the protein expression of Keap1 was down-regulated, the translocation of Nrf2 from the cytoplasm to the nucleus was significantly increased, then the transcriptional activity was increased, and the expression of the downstream HO-1 antioxidant protein was increased, indicating that CBD may improve the cardiac function of exhaustive exercise training mice by activating Keap1/Nrf2/HO-1 signaling pathway. Molecular docking results also confirmed that CBD had a good binding effect with Keap1/Nrf2/HO-1 signaling pathway proteins. In conclusion, the protective mechanism of CBD on myocardial injury in exhaustive exercise training mice may be to activate Keap1/Nrf2/HO-1 signaling pathway, and then exert anti-inflammatory, anti-apoptosis and inhibition of oxidative stress.

## 1. Introduction

The heart is the blood supply organ of the human body. The function of the heart is not only important for maintaining the blood supply, but also for the transport of oxygen, nutrients and metabolites. Long term excessive exercise will cause irreversible damage to the nervous system, immune system, heart, liver and skeletal muscle, and the heart is one of the most sensitive organs affected by overtraining [1]. Excessive exercise training can damage the shape and structure of the heart, which can affect cardiac function, leading to severe arrhythmias, heart failure, reduce exercise capacity, and even the risk of death [2–5]. Due to the different effects of exercise training on myocardium, excessive fatigue and exercise injury have become serious problems affecting the effect of exercise training [3,6–9], so it is of great significance to study exercise-induced myocardial injury and its protection. In recent years, the mechanism and intervention of exercise-induced myocardial injury

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have become an important research direction.

Oxidative stress and inflammatory factors play an important role in the mechanism of exercise-induced myocardial injury. Studies have shown that during high-intensity exhaustive exercise or acute high-intensity exercise, myocardial ischemia and hypoxia will occur temporarily during exercise, and reoxygenation after exercise will induce the generation of a large number of oxygen free radicals, leading to the enhancement of lipid peroxidation, resulting in the abnormal function of biofilm such as mitochondria, resulting in myocardial oxidative damage [6–8]. Studies as well as have confirmed that intensive exercise can lead to a significant increase in pro-inflammatory cytokines such as TNF-α, IL-1β, IL-8, and IL-6, so as to cause lesions and injuries in the body [7–9]. In addition, hypoxia caused by strenuous exercise is easy to induce apoptosis in myocardial tissue. Studies have shown that the accumulation of intracellular reactive oxygen species (ROS) can induce the release of cytochrome *c* from mitochondria, which can activate the apoptotic enzyme caspase-3 and trigger apoptosis, and hypoxia caused by strenuous exercise can easily lead to apoptosis of myocardial tissue cells [10]. In the field of sports medicine, the relationship between myocardial injury and oxidative stress, inflammatory factors and apoptosis has been widely concerned.

The Keap1/Nrf2/HO-1 pathway is a classic antioxidant stress pathway, Keap1 is a dimeric protein composed of 624 amino acid residues, which is mainly located in the cytoplasm, and shuttles between the cytoplasm and nucleus [11]. The Nrf2 is an inhibitor regulated by intracellular Keap1 protein, then the weakening of Nrf2/Keap1 protein interaction leads to the increase of Nrf2 expression. The HO-1 is induced by various stress conditions such as oxidative stress and inflammatory signals, and it is regulated by Nrf2. The expression profile of cellular protective genes under the control of Nrf2 indicates that the defense mechanism of HO-1 driven by Nrf2 has a protective effect on myocardia [12]. The Nrf2 plays an important role in protecting myocardial cells from oxidative stress injury [13,14]. Jin et al. demonstrated that HO-1 had cardioprotective effects on simulated H9C2 cells in vitro [15]. It is suggested that myocardial injury may be closely related to Keap1/Nrf2/HO-1 pathway.

The protective mechanism of myocardial injury has been widely studied, natural products are often used as protective agents for myocardial injury and have good application prospects [16,17]. Cannabidiol (CBD) is a non-toxic main component extracted from cannabis, which has anti-inflammatory, anti-apoptotic and anti-oxidative stress ability. Studies have reported that CBD has a good therapeutic effect on autoimmune myocarditis, which can improve cardiac systolic and diastolic dysfunction, reduce coronary artery contraction, enhance the vasodilation function of mesenteric arteries and improve metabolic parameters [18]. Hao et al. founded that CBD reduced myocardial lipid peroxidation, restored GSH levels and GPx activity, thereby reducing free radical reactions in the myocardium [19]. Studies have shown that cannabidiol can produce anti-inflammatory and analgesic effects, and the mechanism is mainly related to CB1 and CB2 receptors [20]. In addition, cannabinoid can attenuate cardiac dysfunction, oxidative stress and fibrosis in diabetes cardiomyopathy [21]. The structure of CBD is shown in Fig. 1. It is of great significance to clarify the effect of CBD on exercise-induced myocardial injury for the new use of drug CBD and to ensure the heart health of athletes.

CBD is widely used in European and American countries and has a wide application prospect in medicine, health care products, food and other fields. According to the results discussed by the WHO Expert Committee on drug dependence, there was no direct evidence that the use of CBD was dependent [22]. In recent years, European and American countries have made great progress in pharmacological research and drug development and application of marijuana extract CBD with non psychoactive ingredients. The preparation of pure CBD has been used in the treatment of multiple sclerosis and childhood seizures [23]. Therefore, the drug safety of CBD can be guaranteed, but the role of CBD in heart injury caused by exercise training is rarely reported. Therefore, we

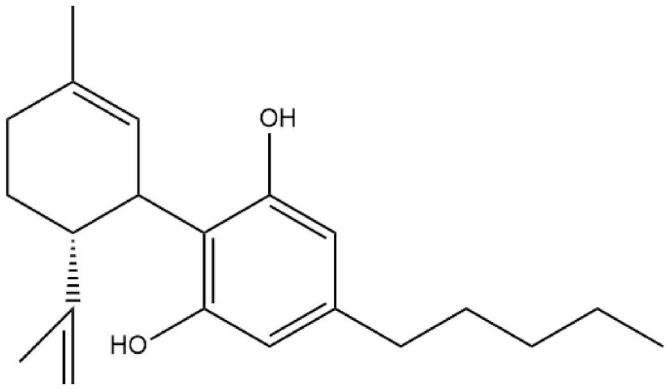


**Fig. 1.** The structure of CBD.

speculate that CBD may play a role in the protection and treatment of exercise-induced myocardial injury. In this sduty, the protective effect of CBD on exercise-induced myocardial injury and its potential mechanism were firstly studied.

## 2. Materials and methods

### 2.1. Materials and chemicals

Cannabidiol, white powder, 95% purity, provided by Shenyang Institute of Metal Research, Chinese Academy of Sciences. The mammalian protein extraction kit and cytoplasmic protein preparation kit, BCA protein concentration determination Kit (BCA reagent, copper sulfate solution, protein standard, protein standard preparation), protease inhibitor and PBS crystals were provided by Hangzhou Ford Biotechnology Co., LTD. Sodium dodecyl sulfonate (SDS), Twin-20 and Twin-80 were purchased from Beijing Solebo Technology Co., LTD. Prestaining protein Marker, SDS-PAGE gel kit (4% and 10% respectively), the ECL chemiluminescence solution, ammonium persulfate (APS) and glycine were obtained from Bio-RAD, USA. TEMED was provided by Beijing Dingguo Changsheng Biotechnology Co., LTD. Methanol, chloral hydrate, anhydrous ethanol and neutral gum were purchased from Sinopharm Chemical Reagent Co., LTD. The high protein skim high calcium milk powder was obtained from Inner Mongolia Yili Industrial Group Co., LTD. HRP labeled goat anti-rabbit secondary antibody, Keap1, Nrf2, HO-1 antibody, IL6, IL10, TNF-α, NF-κB antibody, α-actin, Histone antibody, Bax, Bcl-2, Caspase-3 antibody, IRE-α, COX-2, MDA-5, SOD-1 antibody, HRP labeled goat anti-mouse secondary antibody, DAPI and fluorescent secondary antibody sheep anti-rabbit were purchased from American Abcam company. The immunohistochemical kits and DAB chromogenic kits were obtained from Guangzhou Vickers Biotechnology Co., LTD. The wheat germ agglutinin (WGA) antibodies labeled WITH FITC were purchased from Sigma Company, Germany. The masson staining kits were obtained from Zhuhai Besso Biotechnology Co., LTD.

### 2.2. Animals

The C57BL/6 male mice, aged 6–8 weeks and weighing 18–22 g, were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (license No.: scxk (Liao) 2015–0001). It was kept in the animal room of the trauma Laboratory of the northern theater general hospital and reviewed by the hospital ethics committee. The indoor temperature was kept at 25 ± 1 °C (24-h alternating light and dark). SPF-grade feed and purified water were provided every day. All animals were fed adaptively for one week before modeling. All participants have completed and passed the standardized training of animal experiment and feeding. The ethical approval number was SYYXY2021092001.

### 2.3. Mice grouping

The male C57BL/6 mice (n = 24) were randomly divided into three groups: blank control group (control group, n = 8), exhaustive exercise training group (model group, n = 8), exhaustive exercise training group + CBD dose group (CBD group, 50 mg/kg, twice a week, n = 8). The injection time of CBD solution is 30 min before exercise. The CBD group was given intraperitoneal injection of 50 mg/kg CBD solution (solvent: 1% Tween 80 + 20% ethanol + normal saline). The model group was intraperitoneally injected with the same amount of placebo (1% Tween 80 + 20% ethanol + normal saline), twice a week.

### 2.4. Training program

The control group: no exercise, quiet feeding. The model group and CBD group: mice in each group were subjected to platform adaptation training (10 m/min, 30 min/d, 7 days in total). In formal training, the incline is 10°, the speed is 10 m/min (40%–50%$VO_2$ max) for 10 min, 15 m/min (50%–60%$VO_2$ max) for 10 min, then 20 m/min (78%$VO_2$ max) to exhaustion. Exercise training 6 days per week for 3 weeks.

Exhaustion evaluation criteria: use sound, light, electric stimulation or brush to stimulate the tail of the animal during exercise, and keep the animal in front of the runway as far as possible during exercise, so as to ensure the exercise load. When exercising at the level 3 load, the mice failed to adhere to the running speed of this load and stopped at the rear 1/3 of the runway for more than 3 times successively, with signs exhaustion and ineffective stimulation, which were the criteria for judging exhaustion.

### 2.5. Cardiac ultrasonography

Cardiac ultrasonography was performed 24 h after the end of three weeks of treadmill exercise. After isoflurane anesthesia, the precordial area of mice was depilated with depilatory cream after the lack of activity and anesthetic reaction of mice. The ultra-high resolution small animal ultrasound imaging system vevo2100 and 10 MHz ultrasound probe were used to determine the heart of mice. The left ventricular end-systolic and end-diastolic diameter, the left ventricular ejection fraction and left ventricular fraction shortening, the left ventricular end systolic volume and left ventricular end diastolic volume were measured in mice with M-mode mode.

### 2.6. Sampling and sample preparation

Each mouse was intraperitoneally injected with 2% pentobarbital sodium solution at 0.05 mL/10 g. The mice were anesthetized and killed. The abdominal cavity was exposed, then the thoracic cavity was opened, then the heart was isolated, and the mouse heart was rinsed in pre-cooled normal saline. One third of the heart near the apex was used for protein detection experiments and placed in a refrigerator at −80 °C for subsequent detection, while the rest was fixed with 10% neutral formaldehyde for pathological experiments.

### 2.7. Histopathological analysis

The heart was fixed with 10% neutral formaldehyde, dehydrated, transparent, paraffin embedded, and then made into 4 μm sections. The slices were spread, and baked overnight. After natural drying, the slices were stained with hematoxylin-eosin (HE) and masson staining, and the pathological changes of the heart tissue were observed under a microscope.

### 2.8. Determination of myocardial cell cross-sectional area

The left ventricular portion of the heart tissue was fixed with 10% neutral formaldehyde. Myocardial sections with thickness of 3–5 μm were stained by wheat germ agglutinin (WGA) staining to determine the cross-sectional area of myocardial cells.

### 2.9. Immunohistochemical staining

Paraffin sections were taken for routine dewaxing and dehydration, and antigen was repaired under high pressure. The slices were dropped with 10% sheep serum and sealed in a wet box for 30 min, then NF-κB and COX-2 primary antibody diluent were added and incubated overnight at 4 °C. After washing with PBS for 3 times, the second antibody working solution and peroxidase were added successively, and the slices were incubated at 37 °C for 10 min, then the slices were washed with PBS for 3 times (5 min/time). The slices were dripped with DAB chromogenic solution, counterstained with hematoxylin for 6 min, washed with water, differentiated with hydrochloric acid and ethanol for 3 s, washed with water to turn blue, dried with neutral gum to seal the slices. The immunohistochemical staining of the mouse heart was observed under the microscope. The optical density of the images was analyzed by ImageJ software, and the relative contents of NF-κB and COX-2 were determined.

### 2.10. Western blot

The total protein was extracted from frozen heart tissue by adding cold protein lysate and protease inhibitor. The total protein concentration of each group was determined by BCA kit. The tissue protein was separated by 10% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane. The membrane was washed with TBST (TBS, 0.1% Tween-20) for 3 times and sealed with 5% defatted milk powder at room temperature for 2 h. The membrane was washed with TBST, and the corresponding strip was cut with reference to marker. Next, different antibody diluents (IL6, IL10, TNF-α, Bax, Bcl-2, caspase-3, IRE-α, MDA-5, SOD-1, Cytoplasmic Keap1, Nuclear Nrf2, HO-1, GAPDH, α-actin, Histone) prepared by PBST were added to the membrane and incubated overnight at 4 °C. The membrane was washed 3 times with TBST, 10 min each time. The second antibody reaction solution was added and the membrane was incubated at room temperature for 2 h, then the membrane was washed with TBST for 3 times, 10 min each. The ECL luminescent fluid was used for development and was placed in a chemical gel imaging system for scanning exposure. The Image J software was used to measure the gray values of each strip and each hole, and the corresponding internal parameters were taken as the standard for measurement and analysis.

### 2.11. Molecular docking

The energy of compound CBD was minimized and saved in mol2 format using Chem3D 21.0.0 software. The Keap1 (PDB ID: 6V6Z), Nrf2 (PDB ID: 6T7V) and HO-1 (PDB ID: 1N3U) proteins were downloaded from RSC PDB (https://www. rcsb.org/) database. Then, the protein was dehydrated and hydrogenated by sybyl-X2.0 software, and saved it in PDB format. The interaction between the compound and the target protein was carried out by sybyl-X2.0 software and the molecular docking score was obtained.

### 2.12. Statistical analyses

The experimental data in this study were statistically analyzed by SPSS 26.0 software, expressed by mean ± standard deviation, and statistically analyzed by one-way analysis of variance (ANOVA). When $p < 0.05$, the difference was statistically significant. The statistical charts were obtained by Graphpad Prism 7.0.0 software.

## 3. Results

### 3.1. Effects of CBD on body weight in exercise-induced myocardial injury mice

As shown in Fig. 2, 1–7 days was the adaptive training stage, 8–28 days was the formal experiment stage. The average body weight of mice in the three groups showed an increasing trend, but the body weight decreased on day 2, 9 and 20, which may be caused by exercise stimulation and intraperitoneal injection stimulation, but the fluctuation of body weight was small. IP was the drug delivery node, twice a week, a total of 6 times. The average weight of the control group and CBD group was between 23 and 26 g, and that of the model group was between 23 and 25 g, with no significant difference in body weight. The average weight of the three groups began to differentiate significantly on the 20$^{th}$ day. The model group was lighter than the other two groups, and the average body weight in the last 9 days was control group > CBD group > model group.

### 3.2. Effects of CBD on cardiac function in exercise-induced myocardial injury mice

As shown in Fig. 3, compared with the control group, the left ventricular end-systolic and end-diastolic diameter of mice in the model group was significantly changed ($p < 0.05$), and the left ventricular ejection fraction and left ventricular fraction shortening of mice were significantly decreased ($p < 0.05$). Compared with the control group, the left ventricular end systolic volume and left ventricular end diastolic volume in the model group were increased ($p > 0.05$). Compared with the model group, the left ventricular end-systolic diameter was increased in CBD treated mice ($p < 0.05$). In addition, the left ventricular end systolic volume and left ventricular end diastolic volume in the CBD treatment group were reduced compared with that of the model group ($p > 0.05$).

### 3.3. Effect of CBD on HE staining of myocardial tissue in exercise-induced myocardial injury mice

The results of HE staining were shown in Fig. 4. In the control group, cardiomyocytes were arranged orderly without degeneration, necrosis, swelling and rupture. In the model group, there were swelling of cardiomyocytes, disordered arrangement of cardiomyocytes, rupture of some myocardial fibers and infiltration of inflammatory cells. Compared with the model group, the swelling degree, rupture degree and inflammatory cell infiltration degree of cardiomyocytes in the CBD group were alleviated.

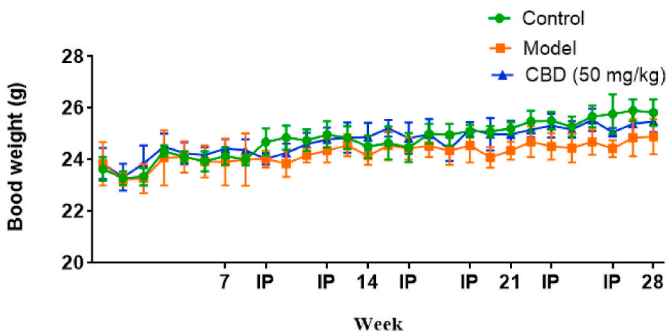


**Fig. 2.** Growth curves of mice. All data are expressed as the mean ± standard deviation (SD) (n = 6). IP is the dosing node, 2 times a week, a total of 6 times. The mice were divided into the control group, the model group and the CBD group (50 mg/kg).

### 3.4. Effect of CBD on myocardial interstitial fibrosis in exercise-induced myocardial injury mice

Masson staining results were shown in Fig. 5. There was no obvious collagen fiber proliferation in the control group. Compared with the control group, the blue staining area in the model group was obvious, indicating that there were a large number of collagen fiber hyperplasia in myocardial tissue and the degree of cardiac interstitial fibrosis was obvious. Compared with the model group, the blue staining area in the CBD group was reduced and the degree of collagen fibrosis was alleviated. It had been confirmed that CBD could alleviate myocardial fibrosis in mice with cardiac injury induced by exhaustive exercise.

### 3.5. Effects of CBD on cross-sectional area of cardiomyocytes in exercise-induced myocardial injury mice

The results of CBD on the cross-sectional area of cardiomyocytes in exhaustive exercise training mice were shown in Fig. 6. Compared with the control group, the cross-sectional area of myocardial cells in the model group was significantly increased ($p < 0.05$). The area of cardiomyocytes in the CBD group was significantly lower than that in the model group ($p < 0.05$). The results indicated that the cross-sectional area of cardiomyocytes was increased significantly in mice after exhaustive exercise training, resulting in myocardial hypertrophy. CBD could inhibit the increase in the cross-sectional area of cardiomyocytes in mice induced by exhaustive exercise training and attenuate cardiac hypertrophy.

### 3.6. Effects of CBD on myocardial inflammation in exercise-induced myocardial injury mice

The inflammatory level of mice heart tissue was shown in Fig. 7 A-C. The expression levels of IL-6, IL-10 and TNF-α in mice heart tissue were analyzed by Western blot. The content of NF-κB was detected by immunohistochemistry. The inflammatory indexes of IL-6, TNF-α and NF-κB in the model group were significantly increased ($p < 0.05$), and the protein expression of IL-10 was significantly decreased compared with the control group ($p < 0.05$). After CBD treatment, the inflammatory indexes of IL-6, TNF-α and NF-κB were significantly decreased ($p < 0.05$), while the protein expression of IL-10 was significantly increased ($p < 0.05$). The content of COX-2 in mice heart tissue was detected by immunohistochemical method (Fig. 7D). The content of COX-2 in the model group was significantly increased ($p < 0.05$) compared to the control group, and there was a large amount of expression around the nucleus. Then the CBD significantly inhibited the expression of COX-2 compared with that of the model group ($p < 0.05$).

### 3.7. Effects of CBD on myocardial apoptosis in exercise-induced myocardial injury mice

Western blot was used to detect the expression levels of apoptosis indicators Bax, Caspase-3 and Bcl-2 in mice heart tissue, as shown in Fig. 8. The protein expression of Bax, Caspase-3 and Bcl-2 in the model group was significantly increased in comparsion to the control group ($p < 0.05$). After the CBD intervention, the expression of three proteins was markedly inhibited in comparison with that of the model group ($p < 0.05$).

### 3.8. Effects of CBD on cardiac oxidative stress parameters in exercise-induced myocardial injury mice

The results were shown in Fig. 9. Compared with the control group, the protein expressions of MDA-5, IRE-1α and NOX-2 in the model group were significantly increased ($p < 0.05$), while the protein expression of SOD-1 was markedly decreased ($p < 0.05$). Compared with the model group, the protein expressions of MDA-5, IRE-1α and NOX-2 were

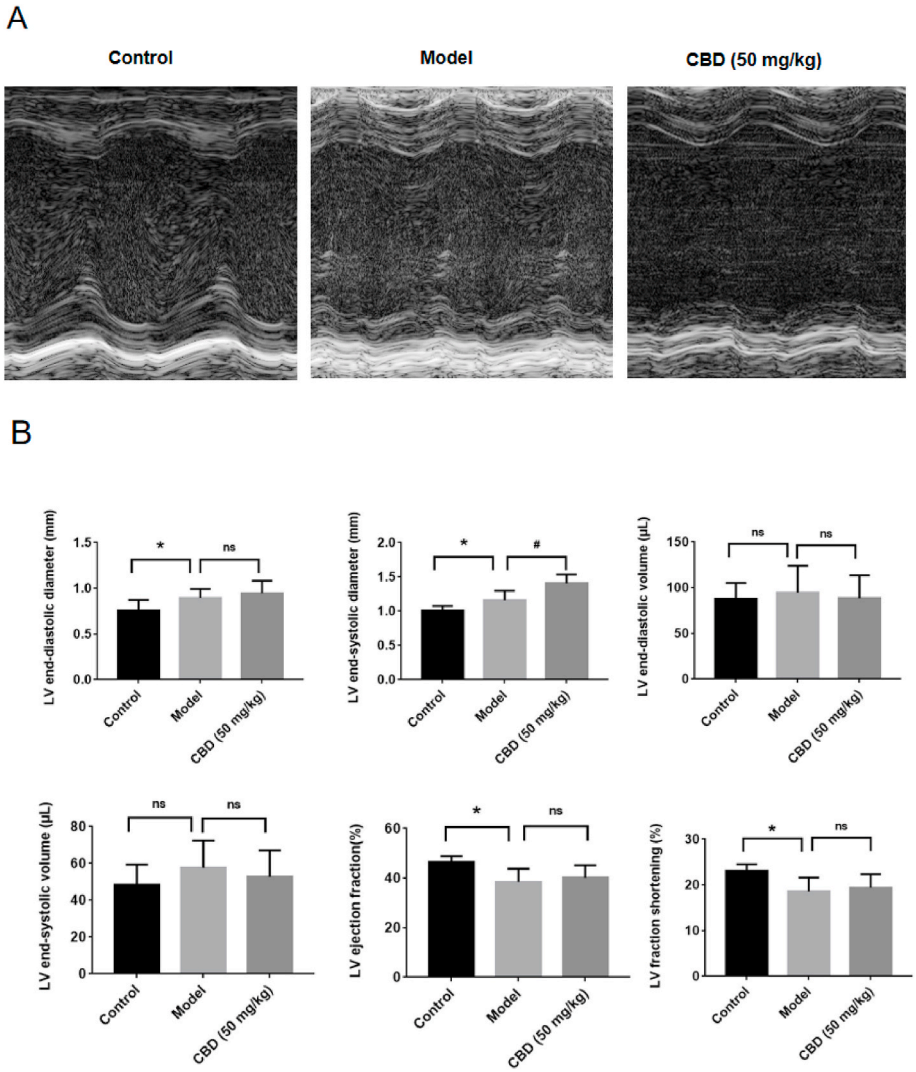


**Fig. 3.** Effect of CBD on cardiac function of exhaustive exercise training mice. Ultrasound images of mice in each group (A), ultrasonic statistics of mice heart (B). The mice were divided into the control group, the model group and the CBD group (50 mg/kg). All data are expressed as the mean ± standard deviation (SD) (n = 6). $^{*}p < 0.05$ compared with the control group. $^{\#}p < 0.05$ compared with the model group. ns: no statistical significance.

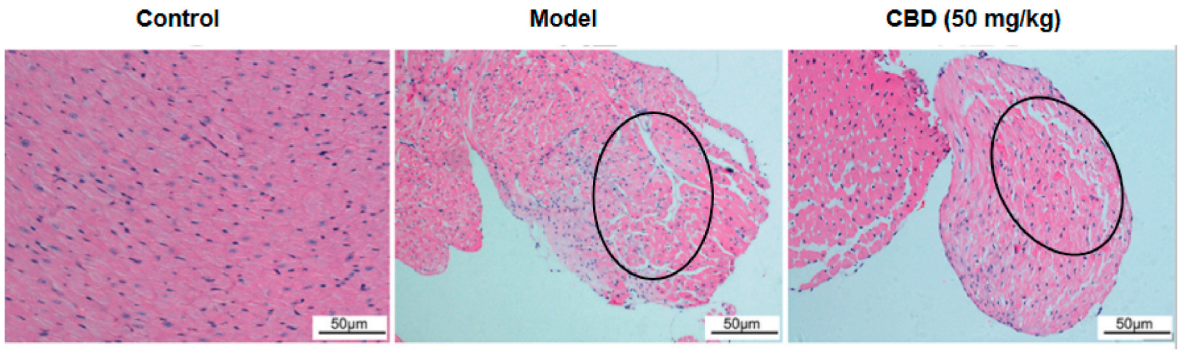


**Fig. 4.** Effect of CBD on HE staining of mice myocardial tissue. Circles: myocardial damage. The mice were divided into the control group, the model group and the CBD group (50 mg/kg).

signally decreased after the CBD intervention ($p < 0.05$). The decrease of SOD-1 protein expression was inhibited by CBD in comparison with that of the model group ($p > 0.05$).

### *3.9. Effects of CBD on Keap1/Nrf2/HO-1 signaling pathway proteins in exercise-induced myocardial injury mice*

The results were shown in Fig. 10. Compared to the control group, the protein expression of Keap1 in the model group was observably increased ($p < 0.05$). The protein expressions of Nrf2 and HO-1 were

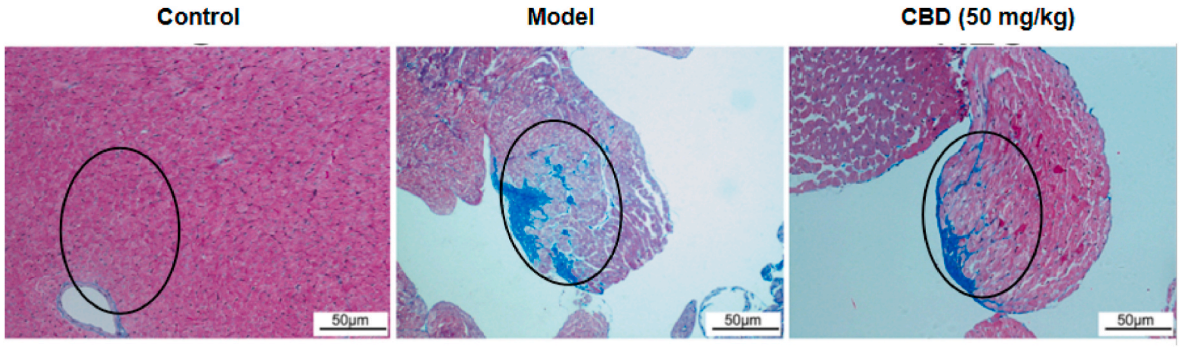


**Fig. 5.** Effect of CBD on myocardial interstitial fibrosis in exhaustive exercise training mice. The Left ventricle of mice sections were stained with masson's trichrome, and micrographs were taken at 200 × magnification. Circles: myocardial fibrosis. The mice were divided into the control group, the model group and the CBD group (50 mg/kg).

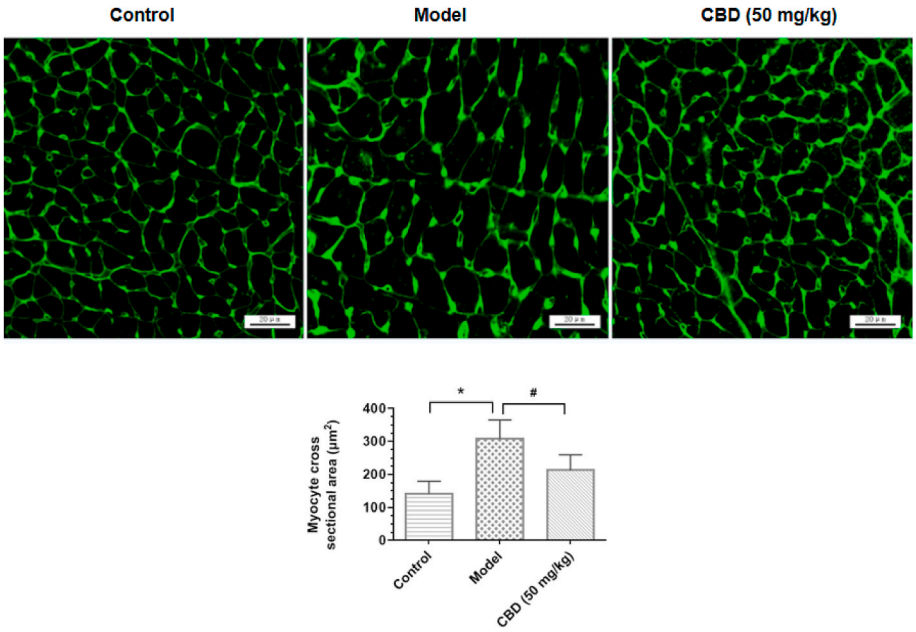


**Fig. 6.** Effect of CBD on cross-sectional area of cardiomyocytes in exhaustive exercise training mice. The micrographs were taken at 400 × magnification. The mice were divided into the control group, the model group and the CBD group (50 mg/kg). All data are expressed as the mean ± standard deviation (SD). $^{*}p < 0.05$ compared with the control group. $^{\#}p < 0.05$ compared with the model group.

significantly decreased compared with that of the control group ($p < 0.05$). Compared with the model group, the protein expression of extranuclear Keap1 were signally decreased after the CBD intervention ($p < 0.05$). The expression of Nrf2 and HO-1 proteins in the CBD group was significantly increased compared to those of mice in the model group ($p < 0.05$).

### 3.10. Molecular docking analysis

The binding between CBD and Keap1/Nrf2/HO-1 proteins was verified based on molecular docking. In the docking results, total_score≥5 and cscore≥3 indicated that the active ingredient had a good binding with the target protein. Total_score >7 indicated that had a strong binding activity. The scoring results of molecular docking were shown in Table 1. The CBD can have good binding with Keap1/Nrf2/HO-1 proteins. The total_score of CBD and Keap1 protein was 6.49, CBD and Nrf2 protein was 6.75, CBD and HO-1 protein was 9.41. The total_scores between CBD and Keap1/Nrf2/HO-1 proteins were all greater than 6, and the cscores between CBD and Keap1/Nrf2/HO-1 proteins were both 4. According to the 3D results in Fig. 11, CBD were all bound in the cavity pockets of the three proteins. According to the 2D results in Fig. 11, the main types of interaction between CBD and Keap1 protein include hydrogen bond and hydrophobic interaction. For example, CBD formed hydrogen bond with the residue SER602. It formed hydrophobic interaction with PHE577, TYR572, TYR334, ALA556 and TYR525. CBD formed hydrogen bonds with Nrf2 protein residues VAL512, VAL418 and ILE416, formed alkyl interactions with VAL606, ALA366 and ALA556, and formed C–H bonds with GLY511. CBD formed hydrogen bonds with HO-1 residues THR135 and HIS25, and formed hydrophobic interactions with ALA28 and PHE207.

## 4. Discussion

Our study mainly explored whether injection of CBD before exhaustive exercise in mice had a protective effect on the heart and its mechanism. The results showed that the body weight, cardiac function and ejection fraction of mice were decreased after exhaustive exercise training. Studies have also demonstrated that exhaustive exercise can cause weight loss in animals, indicating that exercise can induce compensatory thickening of the myocardium [24]. Cardiac pathological examination revealed pathological changes in myocardial structural changes, myocardial hypertrophy, muscle fiber rupture, inflammatory

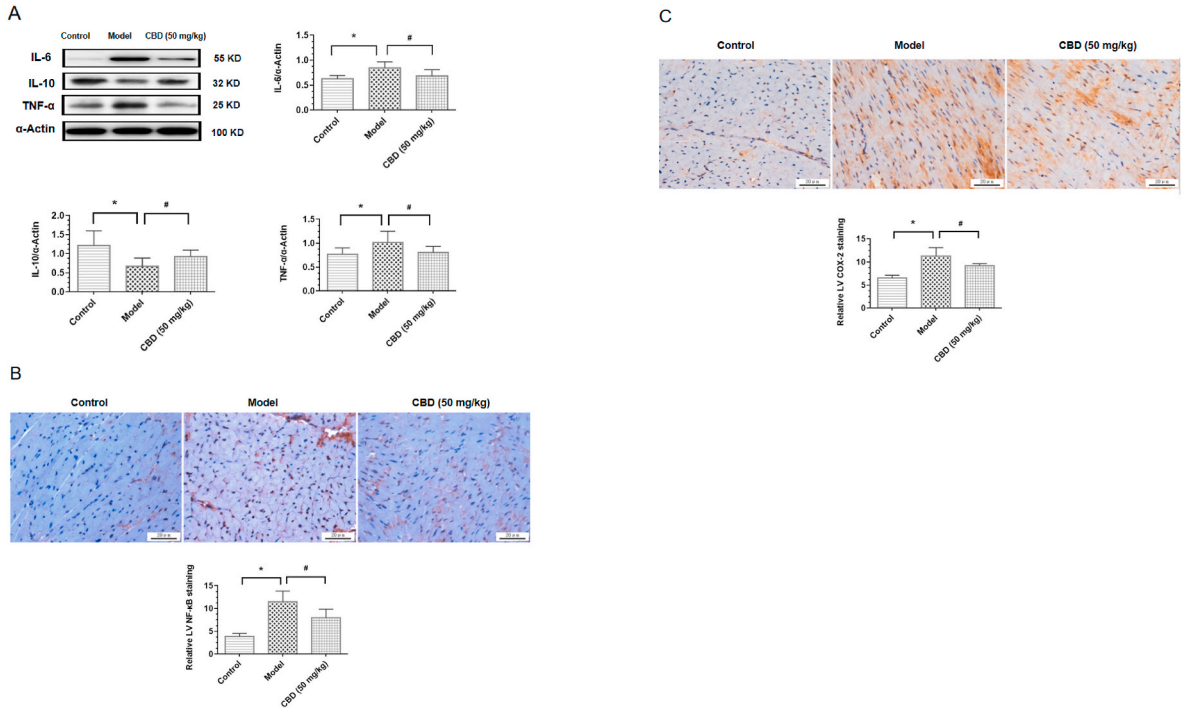


**Fig. 7.** Effects of CBD on myocardial inflammation in exhaustive exercise training mice. The analysis of Western blot for CBD in myocardial cells (A) of exhaustive exercise training mice and quantification of protein expression. The protein levels (IL-6, IL-10 and TNF-α) were normalized to α-actin. Immunohistochemical expression and quantitative data of NF-κB in mice left ventricle (B). The expression of COX-2 was analyzed by immunohistochemistry (C), and the micrographs were taken at 400 × magnification. The mice were divided into the control group, the model group and the CBD group (50 mg/kg). All data are expressed as the mean ± standard deviation (SD). $^{*}p < 0.05$ compared with the control group. $^{\#}p < 0.05$ compared with the model group.

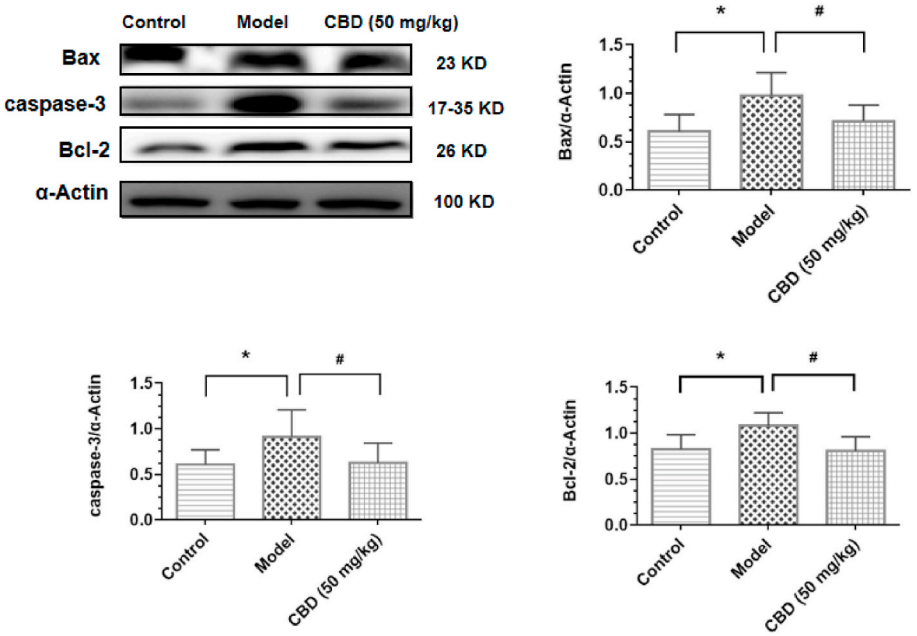


**Fig. 8.** Effect of CBD on myocardial apoptosis in exhaustive exercise training mice. The analysis of Western blot for CBD in myocardial cells of exhaustive exercise training mice and quantification of protein expression. The protein levels (Bax, caspase-3 and Bcl-2) were normalized to α-actin. The mice were divided into the control group, the model group and the CBD group (50 mg/kg). All data are expressed as the mean ± standard deviation (SD). $^{*}p < 0.05$ compared with the control group. $^{\#}p < 0.05$ compared with the model group.

cell infiltration and myocardial fibrosis. It was suggested that exhaustive exercise could reduce the body function and weight of mice, which was also confirmed by the imagination of myocardial hypertrophy. In the Western blot experiment, inflammation, apoptosis and oxidative stress were observed in myocardial tissue. After CBD treatment, the above-mentioned damage was reversed, and it played a protective and therapeutic effect on the mice heart.

Oxidative stress is one of the main mechanisms of myocardial injury. The large production of ROS will lead to the destruction or consumption of antioxidant defense capacity [25,26]. Studies have shown that CBD can inhibit oxidative stress and reduce ROS triggered mitochondrial dysfunction [27]. The results of this experiment indicated that CBD down-regulated the protein expressions of MDA-5, IRE-1α and NOX-2, and up-regulated the expression of SOD-1. It was also confirmed that the improvement effect of CBD on exercise-induced myocardial injury was related to its free radical scavenging effect by enhancing the activity

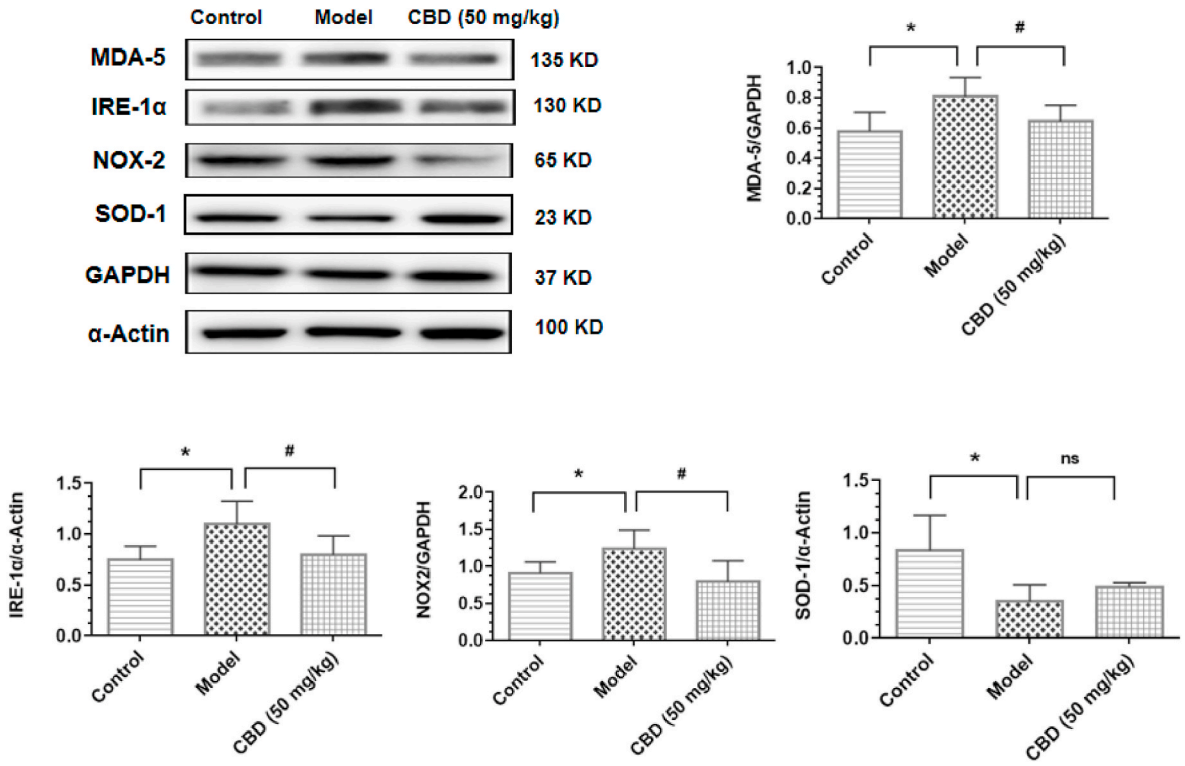


**Fig. 9.** Effect of CBD on myocardial oxidative stress in exhaustive exercise training mice. The analysis of Western blot for CBD in myocardial cells of exhaustive exercise training mice and quantification of protein expression. The protein levels (MDA-5, IRE-1α, NOX-2 and SOD-1) were normalized to α-actin. The mice were divided into the control group, the model group and the CBD group (50 mg/kg). All data are expressed as the mean ± standard deviation (SD). $^{*}p < 0.05$ compared with the control group. $^{\#}p < 0.05$ compared with the model group. ns: no statistical significance.

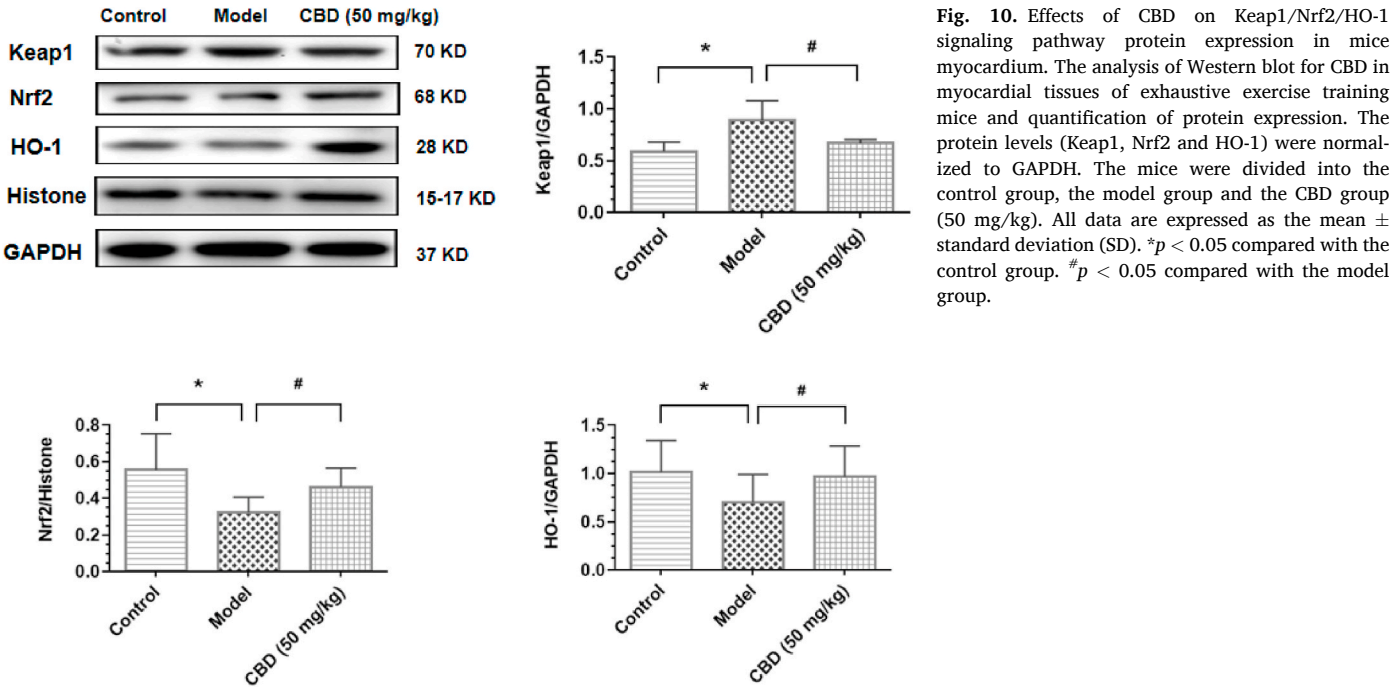


**Fig. 10.** Effects of CBD on Keap1/Nrf2/HO-1 signaling pathway protein expression in mice myocardium. The analysis of Western blot for CBD in myocardial tissues of exhaustive exercise training mice and quantification of protein expression. The protein levels (Keap1, Nrf2 and HO-1) were normalized to GAPDH. The mice were divided into the control group, the model group and the CBD group (50 mg/kg). All data are expressed as the mean ± standard deviation (SD). $^{*}p < 0.05$ compared with the control group. $^{\#}p < 0.05$ compared with the model group.

of antioxidant enzyme. As an important antioxidant enzyme, SOD1 plays a key role in scavenging superoxide free radicals and resisting oxidative stress [28]. MDA-5 is a recognition receptor on cell membrane, and the production of free radicals is closely related to MDA [29]. When NOX family proteins are abnormally expressed, a large number of ROS will be produced. The data of this study also showed that pretreatment with CBD could significantly reduce the expression of Keap-1. Nrf2 in the cytoplasm dissociated from keap-1 and migrated to the nucleus, up-regulated the expression of downstream HO-1, indicating that CBD has a significant anti-oxidative stress effect. These results are consistent

**Table 1**
Docking score of CB with Keap1/Nrf2/HO-1 proteins.

| ligand | Target protein | Molecular docking score | |
|---|---|---|---|
| | | Total score | Cscore |
| CBD | keap1 | 6.49 | 4 |
| CBD | Nrf2 | 6.75 | 4 |
| CBD | HO-1 | 9.41 | 4 |

with those reported in the literature, activation of Keap1/Nrf2/HO-1 signaling pathway can inhibit oxidative stress [30,31]. Nrf2, as a transcription related factor, plays an important role in cell self-protection and is a key regulator of oxidative stress response [32]. Normally, Nrf2 and Keap1 are stably combined in the cytoplasm, when the cell or body is damaged, Keap1 phosphorylation causes Nrf2 to dissociate from Nrf2-Keap1 complex and move into the nucleus, which acts on the HO-1 to regulate the expression of a series of downstream proteins and resist the toxic effect of ROS. This study revealed that CBD may inhibit the expression of oxidative stress proteins MDA-5, IRE-1α and NOX-2 by activating the Keap1/Nrf2/HO-1 signaling pathway, thus effectively resisting oxidative stress injury.

In our study, NF-κB was significantly activated in the cardiomyocytes of mice in the model group, resulting in excessive secretion of inflammatory factors. The intervention of the CBD can inhibit the expression of NF-κB, and then inhibit the inflammatory indexes of TNF-α, IL-6 and COX-2, and promote the expression of IL-10. Consistent with previous research results, the activation of NF-κB can stimulate the expression of TNF-α, IL-6, COX-2 and other inflammatory factors, inhibit the production of anti-inflammatory factor IL-10, and aggravate myocardial injury [33–36]. Studies have shown that inflammation and oxidative stress interact in the cardiovascular system [37]. Continuous oxidative stress leads to the development of inflammation, which inflammation further promotes the production of a large number of oxygen free radicals, resulting in imbalance of the body [37,38]. The inflammatory indexes of NF-κB, TNF-α, IL-6 and COX-2 in the model group were significantly increased, and the expression of anti-inflammatory factor IL-10 was down-regulated, suggesting that the mice in the model group had inflammatory damage. The increased production of free radicals may lead to the development of inflammation [37,38]. In this study, the lipid peroxidation level of MDA-5 was higher compared with that of the control group. The pretreatment group with CBD showed lower level of lipid peroxidation, increased the activity of HO-1/Nrf2, inhibited the expression of NF-κB, TNF-α, IL-6 and COX-2, and slowed down myocardial fibrosis. The results demonstrated that CBD could inhibit myocardial oxidative stress and then inhibit inflammatory response. Studies have confirmed that ulinastatin [39] and catechol [40] can also reduce the secretion of pro-inflammatory factors and inhibit the activation of NF-κB by increasing the level of Nrf2/HO-1 [41].

In this study, the protein expression levels of Bax, caspase-3 and Bcl-2 in the model group were significantly increased, suggesting that the mice in the model group appeared exercise induced cardiomyocyte apoptosis. This study proved that CBD could prevent cardiomyocyte apoptosis under oxidative stress and inflammation. This beneficial effect may be related to CBD inhibiting the overexpression of pro-apoptotic proteins Bax and caspase-3 through Keap1/Nrf2/HO-1 pathway. Previous studies also have shown that both oxidative stress and inflammatory

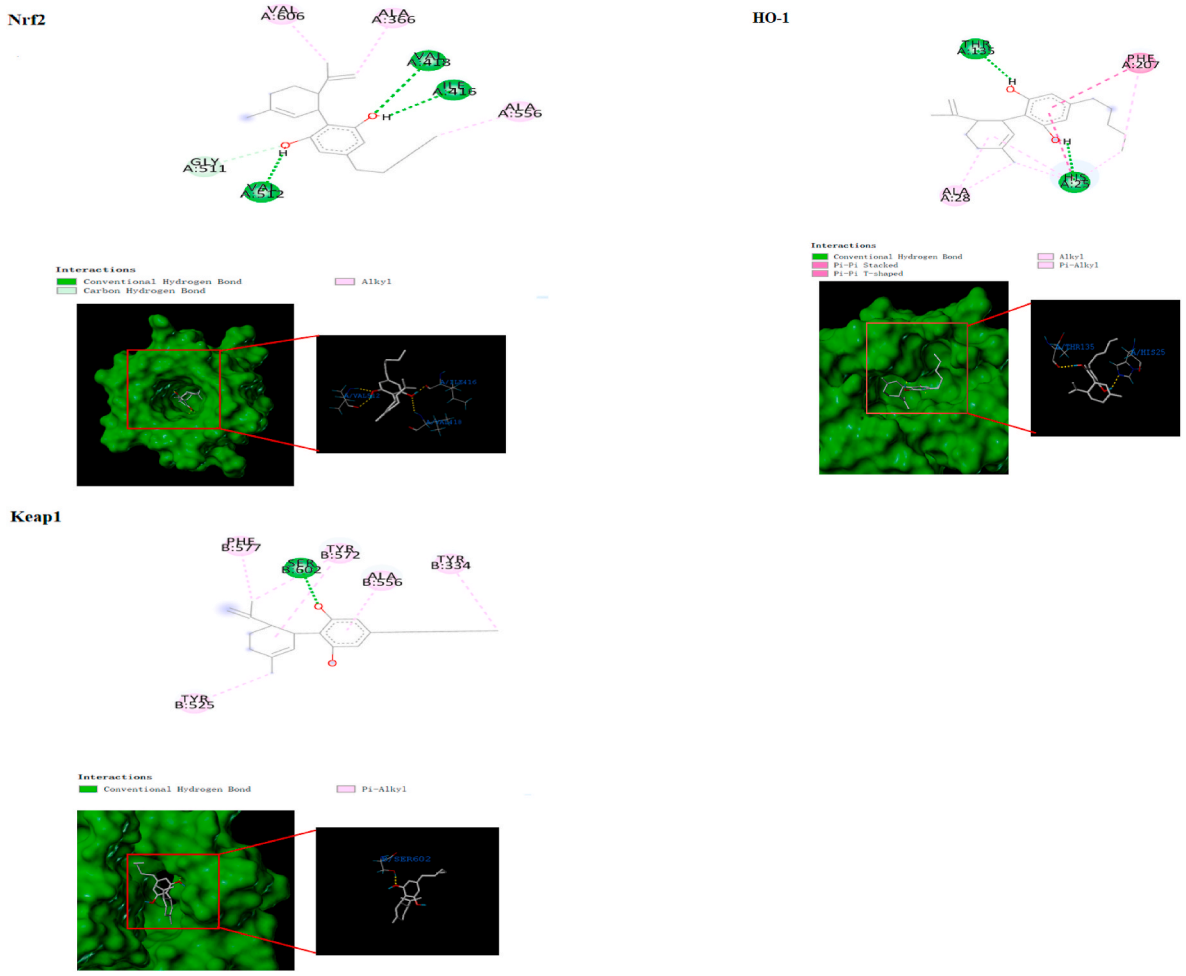


**Fig. 11.** Molecular docking study of CBD binding to Keap1/Nrf2/HO-1 protein.

response can induce apoptosis [42]. HO-1 plays a protective role in oxidative myocardial injury, and HO-1 and its enzymatic hydrolysis product bilirubin resist oxidative stress and inhibit cell apoptosis. It is consistent with our experimental results, Nrf2/keap1 is also involved in regulating the expression level of proteins related to mitochondrial apoptosis pathway and affecting the process of cell apoptosis [43,44]. The study also found that increased expression of Nrf2 can inhibit the expression of Bax protein [45]. The results of this experiment are consistent with literature reports that inflammatory cytokines can also induce cardiomyocyte hypertrophy and apoptosis [46,47].

In addition, molecular docking is a theoretical simulation method to test the binding of small drug molecules and large protein molecules from their respective three-dimensional structures, and predict their binding mode and affinity. Finding a reasonable binding mode between drug molecules and receptor proteins is the basis of molecular docking [48,49]. The molecular docking results in this study indicated that CBD could bind to the catalytic pockets of Nrf2, Keap1 and HO-1, CBD also could form hydrogen bond interaction with active amino acids in Nrf2, Keap1 and HO-1, and CBD could form hydrophobic interactions with the side chains of aromatic amino acids. Therefore, the activation effect of CBD on Nrf2 is to competitively inhibit the combination of Nrf2 and Keap1 by binding to the pocket of Keap1, so as to dissociate Nrf2 from Keap1. Accumulated Nrf2 translocates into the nucleus and binds to HO-1, thereby activating the intracellular antioxidant defense system. These results demonstrated that CBD may be the activator of Nrf2 and HO-1. The molecular docking results were consistent with the results obtained in this experiment, which further indicated that CBD could play a protective role on exercise-induced myocardial injury through the Keap1/Nrf2/HO-1 pathway. There are still some shortcomings in this study, such as no grouping with different doses of CBD and small sample size, etc. The cardioprotective effect of CBD through the regulation of Keap1/Nrf2/HO-1 pathway needs further study.

In conclusion, the above results demonstrated that CBD could reduce oxidative stress injury, inhibit inflammatory release and regulate the expression level of apoptosis related proteins, which may be related to the activation of Keap1/Nrf2/HO-1 signaling pathway, so as to provide experimental basis for the medicinal treatment of exercise-induced myocardial injury by CBD.

## Author statement

No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication and no part of this paper has published or submitted elsewhere.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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